

Mycological Survey and Total Aflatoxin Analyze in Silage from Qaemshahr City (Northern Iran)

M. Hashemi¹, I. Gholampour Azizi², Z. Rezaei¹, S. Rouhi^{1*}

¹Department of Microbiology, Tonekabon Branch, Islamic Azad University, Tonekabon, Iran

²Department of Veterinary Science, Babol Branch, Islamic Azad University Babol, Babol, Iran

Abstract: In this study, 42 samples of silage were collected in Qaemshahr city in Iran during fall 2011. Samples were tested by competitive ELISA for total aflatoxins. Seven samples (16.7%) (Mean±se:1.24) from 42 silage samples were positive with total aflatoxin (1.1-27.3 ppb) in Nov., Dec. and Oct. The highest contamination was observed in Oct., three samples (21.4%) from 14 samples were contaminated with total aflatoxin by 22.2 ppb, 25.6 and 27.3 ppb. The culture results of samples showed that the most toxinigenic fungi among 57 colonies were *Aspergillus sp.* with fifteen (% 31.5), *Fusarium sp.* With thirteen (% 22.8), *Alternaria spp.* With twelve (21.05%), *Penicillium spp.* with nine (%15.78) and *Acremonium sp.* with five (8.77%) and also the most nontoxigenic fungi were *cladosporium spp.* with fifteen (% 37.5), *Rhizopus spp.* with nine (% 22.5), *Mucor spp.* with six (15%), *yeast* with six (15%) and *Scopulariopsis spp.* with four (10%) colonies isolated in culture medium among 40 colonies of nontoxigenic fungi.

Keywords: Mycological Survey, Total Aflatoxin, Silage, Qaemshahr City

INTRODUCTION

Aflatoxins are abundant and most toxic mycotoxins. This toxin can be found in grains such as corn, peanut, cotton, soybean, rice, wheat and dairy products such as milk, butter, eggs and meat [19, 20]. Aflatoxins produce by *Aspergillus flavus* and *A. parasiticus* in favorable conditions of temperature and humidity. Although 20 types of aflatoxin have been identified, only 4 types (B₁, B₂, G₁ and G₂) are main types and contaminate foods [23]. Aflatoxin M₁ (AFM₁) and aflatoxin M₂ (AFM₂) are oxidative metabolites of AFB₁ and AFB₂. These compounds are semi-dihydrofuran or tetrahydrofuran metabolites that bound to coumarin ring and are produced by liver microsomal enzymes or biotransformation of some species of mammals such as dairy cattle that consume contamination foodstuffs with aflatoxins. These

compounds usually discharge from milk, urine and feces. Aflatoxins are harmful for humans and animals such as fish, rodents, cattle, pigs, waterfowl and birds [8]. Aflatoxins are acute toxin, immunosuppressive, carcinogenic, teratogenic and mutagenic compounds. Aflatoxin was listed carcinogen in group I by IACR and WHO [14]. According to FDA, maximum limits of aflatoxin are only 20 ppb and consumption of foodstuff with higher levels of 20 ppb is injurious for animals and causes reduction of milk and feed, weight loss, prolapsed rectum, preterm delivery and liver damage [4, 3]. Silage is the most important and richest food source for ruminants [15]. Many researchers have shown that presence of fungi and aflatoxin in animal's feedstuffs: Simas and his colleagues (2006) in Brazil reported that 33.75% (27/80) of dairy cattle feeding were contaminated

with total aflatoxin by 1-3 μ g/kg. Also in this study *Aspergillus*, *Mucor*, *Rhizopus*, *Fusarium*, and *Penicillium* were isolated and *Aspergillus* species whit frequency 42.5% was more abundant species [24]. Hadizadeh Moalem and his colleagues (2010) in Iran showed that 34.89% (384/134) of feedstuffs were contaminated with aflatoxin B₁ by 0.4-14 μ g/kg [10]. Ghiasian and Maghsoud (2011) in Iran isolated *Penicillium* (23.7%), *Fusarium* (17.5%), *Cladosporium* (9.1%), *Alternaria* (4.3%), *Rhizopus* (3.9%), *Mucor* (3.4%) from cow feeds [9]. Consumption of spotty silage by cattle causes mycotoxicosis and systemic transformations of toxin into the milk, meat and dairy products causes economic damage and different disease in animal and human [19,5]. The purpose of this study was mycological survey and total aflatoxin analyze in silage samples in Qaemshahr city (Mazandaran province, Iran).

MATERIALS AND METHODS

Samples: In this study, 42 silage samples from livestock in Qaemshahr city were collected in spring 2011. Total aflatoxin contamination was analyzed in 14 samples in every month.

Procedure of Extraction: For determination of toxin in the samples, the samples were dried and then were grounded to powder, and then 20g mixed with 100cc of 70% methanol in blender and shacked continuously for 3 min. After settled, the extract was filtered with whatman No.1 [21].

The ELISA test: Total aflatoxins (B₁, B₂, G₁ and G₂) contamination in the samples was measured by competitive enzyme-linked immunosorbent assay (ELISA), by the Agraquant total aflatoxins assay kit (taken from Romer Singapore Company). In first step, 200 μ l of conjugated solutions were added to uncoated- antibody microplate wells and then 100 μ l of each standard solutions and samples extract were added to it. Then 100 μ l of the this contents transferred to coated-antibody microplate wells for 10 min and were incubated at room

temperature (20-25°C) for 30 min. Aflatoxin in samples and control standards competed with enzyme conjugate for binding to the antibody in solid phase. After washing (any unbound enzymes conjugation was removed by washing), After addition 100 μ l of substrate to each well and incubation at room temperature for 5 min, blue color was observed in wells. By using 100 μ l of stop solution, blue color changed into yellow. Aflatoxin concentration and wells absorbance were read by the ELISA reader in 450-630 nm. Information was analyzed by ANOVA using SPSS software package (p<0.05). Toxin concentration in the samples was compared with standard concentration (according to manufacturer's instruction).

Isolation of fungi from silage samples: For isolation of mycoflora in the samples, an aliquot (100 μ l) of each dilution was spread over the surface of a petri dish containing sabouraud's dextrose agar and allowed to set and incubated at room temperature (20-25°C) for 5-7 days, then number of colonies was counted and identified [22,6].

RESULTS

In this study, from 42 samples of silage, 41 samples were contaminated by different amount of total aflatoxin (1.1-27.3 ppb). All of 14 samples in Oct. showed contamination with total aflatoxin and 3(21.4%) samples were contaminated above the limit of FDA, with 22.2 ppb, 25.6 ppb and 27.3 ppb (Acceptable limits of total aflatoxin are 20 ppb according to FDA). Also among 14 samples in Nov. all of them were contaminated and 2 (14.3%) samples were positive in total aflatoxin with 26.7 ppb and 23 ppb. Of 14 silage samples that were collected in Dec., 13 samples showed contamination with total aflatoxin. In this month, 2 samples were contaminated above the limit by 23.4 ppb and 24.3 ppb. Total aflatoxin distribution in silage in different months showed that in Oct.

21.4% and Nov. and Dec. 14.3% of samples were infected by this toxin respectively. Also of 42 silage samples during the three months, 7 (16.7%) samples were contaminated by total aflatoxin more than of maximum limit. Also infection rate in October was more than of other months (Table 1). The screening of potentially toxigenic fungi from the silage culture in Sabouraud's dextrose agar showed that, of the 57 colonies, *Aspergillus* with 15 (% 31.5) colonies was most frequently isolated

genus, followed by *Fusarium* 13 (% 22.8), *Alternaria* 12 (21.05%), *Penicillium* 9 (%15.78) and *Acremonium* 5 (8.78%) colonies. According to results of cultivation nontoxigenic fungi, of 40 colonies, *Cladosporium* species with 15 (% 37.5), *Rhizopus* 9 (% 22.5), *Mucor* 6 (15%), yeast 6 (15%) and *Scopulariopsis* with 4 (10%) colonies respectively, were most nontoxigenic fungi (Table 2).

Table 1: Distribution of total aflatoxin contamination in silage samples

Month	N	Positive (>20ppb)	%	Mean±Se	SD	Max	Min
Oct.	14	3	21.4	2.40	8.98	27.3	1.1
Nov.	14	2	14.3	2.05	7.65	26.7	1.3
Dec.	14	2	14.3	2.12	7.95	24.3	0
Sum	42	7	16.7	1.24	8.04	27.3	0

SE: Standard Error of Mean, SD: Standard Deviation, N: Number, ppb: µg/kg

Table 2: Distribution of toxigenic and non toxigenic fungi in silage samples

Toxicogenic fungi		
Fungi	Frequency	Percent
<i>Aspergillus sp.</i>	18	31.5
<i>Fusarium sp.</i>	13	22.9
<i>Penicillium sp.</i>	9	15.78
<i>Acremonium sp.</i>	5	8.77
<i>Alternaria sp.</i>	12	21.05
Sum	57	100
Non toxicogenic fungi		
<i>Cladosporium sp.</i>	15	37.5
<i>Rhizopus spp.</i>	9	22.5
<i>Mucor spp.</i>	6	15
<i>Scopulariopsis spp.</i>	4	10
Yeast	6	15
Sum	40	100

DISCUSSION

Silage is one of the most important cattle feeds and is one of the main sources of fungi. Fungal growth reduces nutritional value and some mycotoxins such as aflatoxin and allergenic spores have various acute and chronic effects on humans and animals health [22, 1, 27]. Thus reducing of fungal growth and aflatoxin reduction is important [20]. Attitallam and his colleagues (2009) in Libya in 20

samples of animal feed isolated 10 mold genres including: *Aspergillus*, *Penicillium*, *Fusarium*, *Rhizopus*, *Mucor*, *Alternaria*, *Rhizoctonia*, *Pythium*, *Phyllactinia* and *Sacharomyces cerevisiae* [1]. Pereyra and his colleagues (2008) in Argentina from 90 samples of silage isolated *Fusarium* (39%), *Alternaria* (4%), *Geotrichum* (39%), *Eurotium* (% 39), *Mucor* (39%), *Penicillium* (39%) and *Yeasts* (70%) [18]. in our study, *Fusarium*

(22.8%), *Alternaria* (21.05%), *Penicillium* (%15.78), *Mucor* (15%) and *yeast* (15%) were isolated from the samples. Physical properties of sample and characteristics of silo and samples collection, also presence of different species spores and toxin-producer species in different location can be effective on the type and percent of fungi in our study environment in compared to other studies [5, 18, 26]. Khosravi and his colleagues (2008) in Iran reported that the most dominant species that were isolated from animal feed samples, belonged to *Aspergillus* (56%), *Mucor* (17%), *Penicillium* (15%), *Fusarium* (6%), *Cladosporium* (2%) and *yeast* (4%) [11]. Sassahara and his colleagues (2005) showed that of 98 silage samples in Brazil, 17 samples were infected by total aflatoxin [25]. According to our study, 41 samples were infected with different amount of total aflatoxin and 7 samples showed contamination above the standard limit. Suitable conditions for fungi growth and toxin production in various regions is different [12]. Concentrations of aflatoxin in silage or different foodstuff according to the geographical areas, seasonal alterations, climatic, water activity (aW), temperature, pH, moisture and substrate are different [3, 11, 25]. For example aflatoxin rate in cold months is higher than the hot months or *Fusarium* species cannot survive in the low oxygen and low pH. Also most *Penicillium* species grow at low water activity [2, 9]. Some molds, such as *A. flavus* and *A. parasiticus* can easily grow on feeds with moisture 13-18%, and environmental moisture 50 - 60% [2]. In our study, 7 samples show contamination above the standard limit. Low sanitary of raw materials, low quality of feed and presence of toxigenic molds particularly *A. flavus* which produces more aflatoxin in feedstuffs, cause higher level of toxin in the samples [17]. Other factors such as: plant pathogens, time of harvest, plant resistance, stresses, soil salinity, insect attack and mineral deficiency are effective in contamination [12]. Reddy and his colleagues

(2011) in Malaysia showed that in 80 samples of animal feeds *A. flavus* (87%), *A. nigra* (83%), *F. verticillioides* (47%), *F. graminearum* (43%), *F. proliferatum* (42%) , *F. equiseti* (30%) and *Penicillium sp.* (5%) were prevalent fungi in all samples and aflatoxin B₁ was detected in 18 (22.5%) samples (20.6-135 µg/kg) [21]. In our study, %27.16 was contaminated by total aflatoxin. Since some mycotoxins can be produced by more than one mold species or some fungal species produce several types of mycotoxins, amount and type of toxins in diets may be different [15]. Also difference in analytical methods causes disagreement among researcher's results, contamination level of samples and mycotoxin concentrations [25]. Finally, silage and dairy products should be protected for fungi growth and mycotoxins [20]. Several factors such as appropriate methods in agricultural and storage, genetic manipulation of host plant and resistant strains to mold and toxins production, chemicals measures such as fungicides, insecticides and herbicides reduce pollution by fungi and mycotoxins [7, 13]. We hope results in this study can be effective on management, detection, reduction of fungi growth and mycotoxin production in the livestock industry and silage.

REFERENCES

1. Attitallam, I.H., L.K.T. Al-Ani, M.A. Nasib, I .A.A. Balal, M. Zakaria, S.S.M. El-Maragh and S.M.R. Karim, 2010. Screening of Fungi Associated Commercial Grains and Animal Feeds in AL-Bayda Governorate, Libya. World Applied Sciences Journal., 9, 746-756.
2. Ayar, A., D. Sert and A. Hilmi Con, 2007. A Study on the Occurrence of Aflatoxin in Raw Milk Due to Feeds. Journal of Food Safety., 27, 199-207.
3. Charoenpornsook, K and P. Kavisarasai, 2006. Mycotoxins in Animal Feeds Stuffs of Thailand. Kmitl Sci Tech J., 6(1), 25-28.

4. Devi Upadhaya, S., M.A. Park and J. K. Ha, 2010. Mycotoxins and Their Biotransformation in the Rumen: A Review. *Asian-Aust J Anim Sci.*, 23(9): 1250 – 1260.
5. Driehuis, F., M.S. Spanjer, J.M. Scholten and M.C. Giffel, 2008. Occurrence of Mycotoxins in Feedstuffs of Dairy Cows and Estimation of Total Dietary Intakes. *J Dairy Sci.*, 91(11), 4261-427.
6. El-Shanawany, A.A., M. Eman Mostafa and A. Barakat, 2005. Fungal Populations and Mycotoxins in Silage in Assiut and Sohag Governorates in Egypt, With a Special Reference to Characteristic Aspergilli Toxins. *Mycopathologia.*, 159, 281–289.
7. Fandohan, P., K. Hell, W.F.O. Marasas and M.J. Wingfield, 2003. Infection of Maize by *Fusarium* Species and Contamination with Fumonisin in Africa. *Afr J Biotechnol.*, 2(12), 570-579.
8. Gholampour Azizi, I and M. Azarmi, 2009. Determination of Zearalenone and Ochratoxin in Food stuffs. *World Appl Sci Journal.*, 7(11), 1388-1391.
9. Ghiasian, S.A and A.H. Maghsood, 2011. Occurrence of aflatoxigenic fungi in cow feeds during the summer and winter season in Hamadan, Iran. *Afr J Microbiol Res.*, 5(5), 516-521.
10. Hadizadeh Moalem, S.H., I. Gholampour Azizi and M. Azarmi, 2010. Prevalence of Aflatoxin B₁ in Feedstuffs in Northern Iran. *Global Veterinaria.*, 4(2), 144-148.
11. Khosravi, A.Z., M. Dakhili and S. Hojjatollah, 2008. A Mycological Survey on Feed Ingredients and Mixed Animal Feeds in Ghom Province, Iran. *Pak J Nut.*, 79(1), 31-34.
12. Krnjaja, V., J. Levic and S. Stankovic, 2009. Ubiquity of Toxigenic Fungi and Mycotoxins in Animal Feeds in Republic of Serbia. *J Biotechnol Anim Husbandry*, 25(5-6), 477-497.
13. Kabak, B., 2010. Mycotoxins in Food, Feed and Bioweapons: Prevention and Management of Mycotoxins in Food and Feed, in: Rai, M., Varma.A. (Eds.), MI: Department of Food Engineering, Faculty of Engineering, Hitit University, Corum, Turkey: 201-227.
14. Moktabi, S., A. Fazlara, M. Ghorbanpour and K. Ghasemian Yadegari, 2011. Contamination of ice cream by aflatoxin M₁ in Iran. *American-Eurasian J Toxicol Sci.*, 3, 120-123.
15. Michelle, S., D.V.M. Mostrom and B.J. Jacobsen, 2010. Veterinary Clinics of North America: Food Animal Practice. Ruminant Mycotoxicosis., 27, 315-34.
16. Mansfield, M.A and G.A. Kuldau, 2007. Microbiological and Molecular Determination of Mycobiota in Fresh and Ensiled Maize Silage. *Mycologia.*, 99(2), 269–278.
17. Martins, H.M., M.M.M. Guerra and M.A. F Bernardo, 2007. Occurrence of Aflatoxin B₁ in Dairy Cow's feed over 10 Years in Portugal (1995-2004). *Rev Iberoam Micol.*, 24, 69-71.
18. Pereyra, C.M., V.A. Alonso, C.A.R. Rosa, S.M. Chiacchiera, A.M. Dalceroad and L.R. Cavaglieri, 2008. Gliotoxin Natural Incidence and Toxigenicity of *Aspergillus Fumigatus* Isolated From Corn Silage and Ready Dairy Cattle Feed. *World Mycotoxin J.*, 1(4), 457-462.
19. Richard, J.L, 2007. Some Major Mycotoxins and Their Mycotoxicoses—An Overview. *Int J Food Microbiol.*, 119, 3–10.
20. Rahimi, E, 2010. A study on contamination of Aflatoxin M₁ in milk and infant milk products in Iran. *American-Eurasian J Toxicol Sci.*, 2, 109-111.
21. Reddy, K.R.N and B. Salleh, 2011. Co-Occurrence of Moulds and Mycotoxins in Corn Grains Use for Animal Feed Malaysia. *J Anim Vet Adv.*, 10(5), 668-673.
22. Richard, E., N. Heutte, L. Sage, D. Pottier, V. Bouchart, P. Lebailly and D. Garon, 2007.

- Toxigenic fungi and mycotoxins in mature corn silage. *Food Chem Toxicol.*, 45, 2420–2425
23. Sherif, S.O., E.E. Salama and M.A . Abdel-Wahhab, 2007. Mycotoxins and Child Health: The Need for Health Risk Assessment. *Int J Hyg Environ. Health.*, 1-22.
24. Simas, M.M.S., M.B. Botura, B. Correa, M. Sabino, C.A. Mallmann, T.C.B.S.C. Bitencourt and M.J.M. Batatinha, 2006. Determination of fungal microbiota and mycotoxins in brewers grain used in dairy cattle feeding in the State of Bahia, Brazil. *Food Control.*, 1-5.
25. Sassahara, M., D. Pontes Netto and E.K. Yanaka, 2005. Aflatoxin Occurrence in Foodstuff Supplied to Dairy Cattle and Aflatoxin M₁ in Raw Milk in the North of Parana State. *Food Chem. Toxicol.* DOI:10.1016/j.fct.2005.02.003
26. Wang, J., X. Wang, Y. Zhou, L. Du and Q. Wang, 2010. Fumonisin Detection and Analysis of Potential Fumonisin-Producing *Fusarium* spp. in Asparagus (*Asparagus officinalis* L.) in Zhejiang Province of China. *J Sci Food Agri.* DOI: 10.1002/jsfa.3893
27. Zain, M.E, 2011. Impact of Mycotoxins on Humans and Animals., *J Saudi Chem. Society.* DOI:10.1016/j.jscs.2010.06.006